Thermal (in)stability of type I collagen fibrils.

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We measured Young's modulus at temperatures ranging from 20 to 100°C for a collagen fibril taken from rat's tendon. The hydration change under heating and the damping decrement were measured as well. At physiological temperatures $25-45$°C Young's modulus decreases, which can be interpreted as instability of collagen. For temperatures between $45-80$°C Young's modulus first stabilizes and then increases with decreasing the temperature. The hydrated water content and the damping decrement have strong maxima in the interval $70-80$°C indicating on complex inter-molecular structural changes in the fibril. All these effects disappear after heat-denaturating the sample at 120°C. Our main result is a five-stage mechanism by which the instability of a single collagen at physiological temperatures is compensated by the interaction between collagen molecules within the fibril.

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Type I collagen is the major structural element in the extra-cellular matrix. The native state of collagen is made up by three polypeptide chains, which are twisted together into a triplex [1]. Naively, the collagen triplex is expected to be very stable, since it forms fibrous connective tissues of bones, skin and tendons; see Fig. 1 for the tendon hierarchical structure. But in contrast, the collagen triplex denaturates into separate chains at the helix-coil transition $T_{\rm hc}$, which for mammals and birds is close to [2, 3]—or even lower from [4]—the body temperature (for poikilotherms $T_{\rm hc}$ relates to the upper environmental temperature). Much attention was devoted to the physiological meaning of this marginal thermal (in)stability [3, 4, 5], which is generally explained as compromising between the instability of a single collagen versus flexibility of collagen fibrils [4, 5]. However, it is so far not clear by which specific mechanisms marginally instable collagen molecules achieve to form the stable collagen fibril.

We approach to this problem by studying thermal denaturation of a collagen fibril via its mechanical characteristics such as Young's modulus, logarithmic decrement of damping, and the hydrated water content. The Young's modulus of collagen triplexes and fibrils was measured via various methods [6, 7, 8]. The hydrated water was found to be essential for maintaining the triplex [9, 10]. It is also believed to be important for the structure of fibril, though direct experimental evidences for this are lacking [10]. Combining these quantities enables us to contrast features of a single collagen molecule to those of a fibril, and study the impact of the intermolecular interaction on the fibril stability. The structural transformations in the fibril are determined by competition between entropy increase versus the intermolecular interactions [7]. It is known that for such materials the mechanical properties can provide information not only on internal elastic forces, but also on the molecular

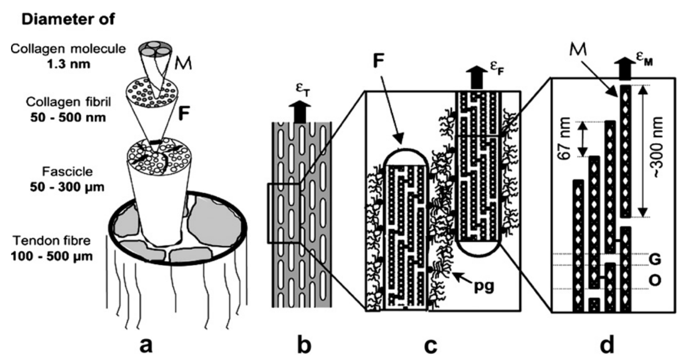

FIG. 1: (**a**) Hierarchic tendon structure: collagen triplexes (M), fibrils (F), fascicle and fiber. (**b**) Fibre is composed of fascicles. (**c**) The fascicle is a composite of collagen fibrils in a proteoglycan-rich matrix (pg). (**d**) The fibril structure: triplehelical collagen molecules (M) are staggered with an axial spacing of 67 nm. There is a succession of gap (G) and overlap (O) zones. The triplexes are stabilized in the fibril by intermolecular cross-links, direct hydrogen bonds and water-mediated hydrogen bonds. $\varepsilon_{\rm T}$, $\varepsilon_{\rm F}$ and $\varepsilon_{\rm M}$ denote strains on the tendon, fibril and separate molecule [14].

processes involving stress relaxation [11]. In particular, these properties can uncover relaxation processes both in the main polymer chain and side groups, e.g., they revealed the phenomenon of low temperature glass transition in the surface layers of protein molecules [12].

We shall study the viscoelastic properties of collagen fibrils for a wide temperature range 20 to $100°C$ and at frequencies within the eigen-frequency domain ($50-20000$ Hz). The collagen fibril is composed of collagen triplexes and hydration water with a small amount of salts. Fig. 1 shows a simplified scheme of the collagen hierarchy from the separate triplex to the tendon. Our mechanical methods allow studying samples with diameter 1 μm, and we shall work with a separate fibril (denoted by F in Fig. 1). See Refs. [13] for optical methods

of studying the collagen structure.

Materials and Methods. Achilles tendons of young rats were obtained from the Yerevan Medical Institute. Separation of fibrils from the fiber and from each other was carried out mechanically in 96% of ethyl alcohol at temperature 5°C using micro-tweezers and microscope. The experimental sample is a cylinder of length 0.3 mm, which is cut-off from a separate fibril, held in the micro-tweezers and washed out in distilled water before experiments. More details on the preparation of similar experimental samples are found in [12].

The sample under investigation was enclosed in the experimental chamber and placed in a temperature-controlled cabinet with the temperature maintained at 25°C. The hydration level of the sample was adjusted by placing a drop of $CaCl_2$ solution at the bottom of the experimental chamber. The sample was allowed to equilibrate at a given humidity for several hours. The relative humidities from 97 to 32% in the chamber were achieved by means of $CaCl_2$ solutions of different concentrations, while the relative humidities 15% and 10% were obtained via saturated solutions of $ZnCl_2$ and $LiCl$, respectively. The chamber was then covered by the heat-insulating jacket and placed on the table of the microscope used to measure the sample vibration. The viscoelastic properties or the sample length were measured point by point when varying the temperature continuously at a rate of 1° C/min. For checking the features of hysteresis and irreversibility (see below) the heating rate was occasionally reduced to 0.1°C/min.

For measuring the Young's modulus E (defined as the ratio of stress [pressure] over strain) and the logarithmic damping decrement ϑ we applied Morozov's micromethod [16]. The method is based on the analysis of electrically excited transverse resonance vibrations of the sample (fibril cylinder), which is cantilevered from one edge (another edge is free). Modifications of the method that enable measuring E and ϑ within a wide temperature range is described in [12, 17].

As a characteristic of internal friction we employ the logarithmic decrement of damping

$$\vartheta = \ln[\,A(t)/A(t+T)\,], \tag{1}$$

where the oscillation amplitude $A(t)$ of the sample has two consecutive peaks at times t and $t+T$. For measuring E and the phase-frequency or the amplitude-frequency characteristics of oscillations (employed for obtaining ϑ), it is necessary to change smoothly the frequency f of the induced oscillations and determine the basic resonance frequency, which corresponds to the maximal oscillation amplitude of the sample free end. Young's modulus for sample main axis is calculated by following formula [15]

$$E = 3.19 \cdot f_0^2 \cdot l^4 \cdot \rho \cdot P/I_{\min}, \tag{2}$$

where f_0 is resonance frequency, l is the sample length, P is the cross-section area, ρ is the density, and $I_{\min}$ is the main inertia moment of that section, which corresponds to the deformation plane with the minimal stiffness. For the round cross-section of our samples $I_{\min} = \pi \cdot D^4/64$ [15] and $P = \pi \cdot D^2/4$, where D is the sample diameter (measured with precision 0.02 μm). Thus Young's modulus is calculated from (2), where l, ρ, P and $I_{\min}$ are the known sample characteristics and f_0 is measured on the experiment.

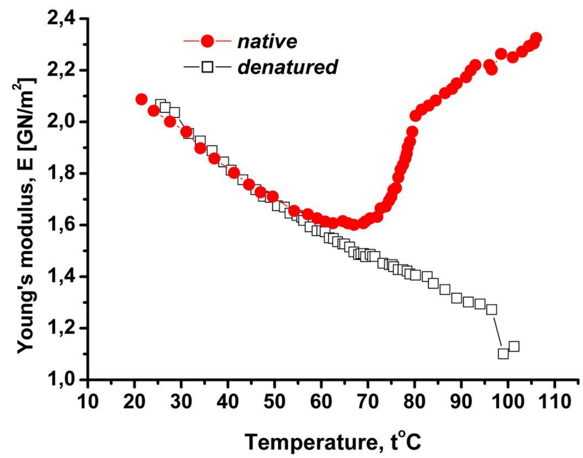

FIG. 2: Young's modulus E versus temperature for the native and heat-denatured collagen fibrils. The heating speed is 1°C/min. Red arrows and indices $1-5$ indicate on temperature intervals discussed in the text.

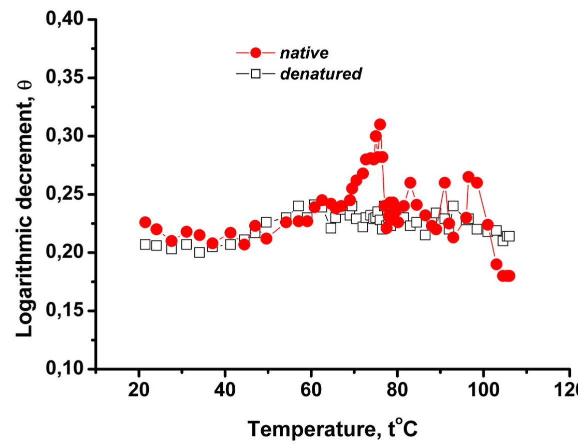

FIG. 3: The logarithmic damping decrement ϑ versus temperature for the native and heat-denatured collagen fibrils. The heating speed is 1°C/min. The red arrow and indices 3 and 4 correspond to those in Fig. 2.

The data on the hydration water content is obtained by measuring the sample mass via the method of [20]. This method allows to detect the changes of mass within 0.00001 mg in microsamples weighting up to 0.01 mg.

Results and Discussions. The main contribution to Young's modulus of collagen fibril comes from the rigid-

ity of separate collagen molecules and from the intermolecular interactions. It was argued recently that the interplay between these two mechanisms is the key for understanding the collagen mechanics [18].

Fig. 2 displays the Young modulus of the collagen fibril versus temperature. At the initial temperature 25°C we created relative humidity 93%. The water content in the fibril is 0.3 g H_2O/g dry collagen.

For the considered frequency range (50 − 20000 Hz) the studied quantities (e.g., Youngs modulus) does not show any significant dependence on the frequency (not shown on figures). Thus we are in the slow deformation regime, e.g., the values of Young's modulus shown in Fig. 1 are consistent with earler results in this regime [6, 7, 8]. It is however expected that dependence on the frequency will show up for larger frequencies (e.g., the Young's modulus starts to increase with frequencies) [6, 7]. For our samples this dependence starts above 100 KHz. In the studied frequency range the hydrated water does not contribute directly to the Young's modulus [19, 20], but the water can induce structural changes in the fibril that will alter its elastic features.

As suggested by our results, the studied temperature domain 20 − 100°C should be separated into five intervals; see Fig. 2. For each of these intervals we discuss the behavior of the measured quantities for the native collagen fibril sample and compare it with the corresponding heat-denaturated sample, which was prepared by keeping the native sample at 100°C for 30 minutes.

1. Young's modulus of the native collagen smoothly decreases between 20°C and 45°C; see Fig. 2 (temperature borders of the intervals are defined conventionally). There is no difference between the Young modulus of the native sample and that of the heat-denaturated sample. In this temperature range the logarithmic decrement of damping (LDD)—which characterizes internal friction and is generally rather susceptible to inter-molecular interactions—does not experience any systematic change; see Fig. 3. The hydrated water content also does not change in this interval; see Fig. 4.

Note that the conformational changes in this interval are completely reversible, since the features of the fibril did not change after repeating the cooling-reheating process ten times. Though the Young modulus and the LDD of the native fibril are almost indistinguishable from those of the heat-denaturated fibril, the hydrated water content (HWC) does show certain differences between the native and heat-denaturated sample; see Fig. 3.

It is likely that in this stage only the triplex conformation changes. We think that the decrease of Young's modulus in this temperature interval is a mechanic analogue of very slow single triplex thermal instability, which was calorimetrically observed in [4]. In one way or another, similar instabilities are seen by many experiments that work at very low concentration, so that the intermolecular interactions do not play any role [2].

2. In the temperature interval 45 − 58°C the decrease of Young's modulus for the native sample is impeded as compared to the previous stage. Now differences between the native and heat-denaturated situations set in: the Young modulus of the native sample is larger and decreases slower as compared to that of the heat-denaturated sample. Also in this interval we noted first indications of hysteresis and irreversibility during heating and recooling (not shown on figures). We checked and confirmed these indications by changing the heating rate from 1°C/min to 0.1°C/min.

Presumably already in this temperature interval the intermolecular interactions influence on the change of Young's modulus. Recall that the collagen triplexes in the fibril are tied with cross-links, direct hydrogen bonds and water-mediated hydrogen bonds; see Fig. 1 (**d**).

3. The third interval lies in 58 − 75°C. Here the Young modulus of the native sample is nearly constant. To our knowledge such an effect was never seen for a single collagen triplex. The Young modulus of the heat-denaturated sample continues to decreases following the same linear law as for the previous stages; see Fig. 2. The logarithmic decrement of damping (LDD) and the hydrated water content (HWC) of the native sample increase suddenly. The endpoint of the interval (approximately 75°C) plays a special role, since here Young's modulus starts to increase, while both LDD and HWC assume their maximal values; see Figs. 3 and 4. In this interval the hysteresis is more pronounced than for the previous interval.

Altogether, this seems to indicate that the intermolecular interactions start to play an important role. Most likely reason for the sudden changes of LDD and HWC—which we stress are clearly absent for the heat-denaturated sample—is that partially molten collagen molecules start to overlap and create new bonds between each other. This facilitates intermolecular interactions. The hydrated water content increases, since new adsorption centers open up during the melting. A similar correlations between the presence of water and the strength of inter-molecular interactions was obtained in Ref. [6] via atomistic modeling.

Note that theoretical arguments predicted recently that the energy dissipation in the collagen fibril gets maximized at the transition from homogeneous intermolecular shear to slip pulses [18]. In our Figs. 2, 3 and 4 we also see simultaneous indications of structural changes (Young's modulus and hydrated water content) and dissipation maximization (LDD pick).

4. Between 75 − 80°C Young's modulus of the native sample starts to increase in sharp contrast to Young's modulus of the heat-denaturated sample that keeps decreasing; see Fig. 1. Simultaneously, both LDD and HWC start to decrease; see Figs. 3 and 4. At the end of this interval (i.e., at 80°C) Young's modulus almost approaches its initial value at 25°C.

We think that a possible reason for increasing Young's

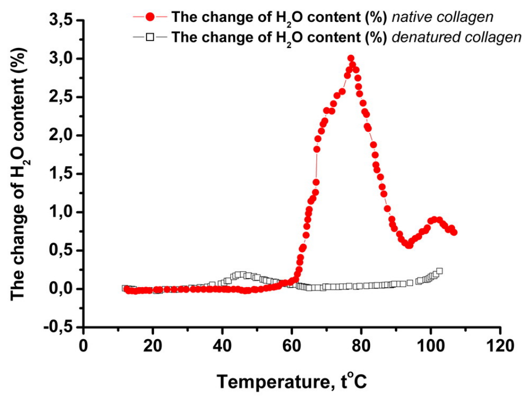

FIG. 4: The hydrated water content (HWC) versus temperature for native and heat-denatured collagen fibrils. The initial temperature and water content in the chamber were 25°C and 93%, respectively. At this temperature and humidity the water content of the native and denatured micro-fibril is $h = 0.3\,\mathrm{g}\,H_2O/\mathrm{g}$ dry collagen and $h = 0.22\,\mathrm{g}\,H_2O/\mathrm{g}$ dry collagen, respectively. The change of the water content is given in percents relative to that water content at 25°C. The heating speed is 1° C/min.

modulus is that the network of the inter-molecular bonds (established already during the previous stage) develops and contributed significantly to the rigidity. This can also expel the water out of the fibril.

We see that the crucial difference between the measured characteristics of the native and heat-denaturated sample indicate on the existence of an important structural feature of the native fibril, which ensures its stability for temperatures larger than 58°C and which is absent for the heat-denaturated sample.

5. Above 80°C the Young modulus keeps on increasing, though slower than for the previous step. The LDD stops decreasing and starts to change irregularly, in contrast to the LDD of the heat-denaturated sample. The HWC in this region keeps on decreasing before 92°C, and then changes non-monotonously. The dynamics in this region is irreversible: if the heating stops at some temperature larger than 80°C and the sample is cooled back to 20°C, the above behavior of the native fibril is not recovered upon subsequent heating. Instead we obtain the heat-denaturated behavior displayed on Figs. 2–4. On the other hand, if the heating simply stops at some temperature larger than 80°C, the sample slowly relaxes to the heat-denaturated value of the Young modulus.

It is likely that the origin of this irreversibility is mainly entropic: there are already so many well-established inter-molecular bonds in this regime that the reverse transition to the weakly coupled inter-molecular situation becomes impossible. The HWC and LDD in this interval behave non-monotonously indicating on further structural changes, which are again absent for the heat-denaturated sample.

In conclusion, we studied thermal stability of the (type I) collagen fibril via measuring its Young's modulus, logarithmic decrement of damping (LDD) and hydrated water content (HWC). All the measurements were done in parallel for the native collagen fibril from rat's tendon and its heat-denaturated version. We aimed to understand how the instability of a single collagen triplex is assimilated by this structure. We observed that between 20°C and 50°C the Young modulus of the native fibril decays with increasing the temperature. This indicates on the (partial) instability of the fibril due to single-collagen effects. For higher temperatures the LDD and HWC indicate on serious structural changes in the fibril. Due to these changes Young's modulus first becomes constant and then (upon further heating) increases with temperature. None of these effects is seen on the heat-denaturated fibril, which displays monotonously decaying Young's modulus and a relatively trivial behavior for LDD and HWC.

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